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Mechanical stimulation by osmotic and hydrostatic pressure activates *Drosophila* oocytes *in vitro* in a calcium-dependent manner

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Abstract

Embryogenesis in vertebrates and marine invertebrates begins when a mature oocyte is fertilized, resulting in a rise in intracellular calcium (Ca^{2+}) that activates development. Insect eggs activate without fertilization via an unknown signal imparted to the egg during ovulation or egg laying. One hypothesis for the activating signal is that deformation of eggs as they pass through a tight orifice provides a mechanical stimulus to trigger activation. Ovulation could produce two forms of mechanical stimulus: external pressure resulting from the passage of oocytes from the ovary into the narrow oviducts, and osmotic pressure caused by hydration-induced swelling of the oocyte within the oviducts. Ovulation could also trigger activation by placing the oocyte in a new environment that contains an activating substance, such as a particular ion. Here, we provide the first evidence that *Drosophila* oocytes require Ca^{2+} for activation, and that activation can be triggered *in vitro* by mechanical stimuli, specifically osmotic and hydrostatic pressure. Our results suggest that activation in *Drosophila* is triggered by a mechanosensitive process that allows external Ca^{2+} to enter the oocyte and drive the events of activation. This will allow exploitation of *Drosophila* genetics to dissect molecular pathways involving Ca^{2+} and the activation of development.

Keywords: Egg activation; Calcium; Mechanosensation; Pressure; French press; Gadolinium

Introduction

Mature oocytes require an external signal to begin development. This signal, which differs among animals, 'activates' the oocyte to resume and complete meiosis, modify its outer coverings, reorganize its cytoskeleton, and translate or degrade certain maternal mRNAs. In most animals, activation is triggered by fertilization, but changes in the ionic environment, changes in pH, or mechanical deformation can initiate egg activation in some species (Harada et al., 2003; Horner and Wolfner, 2008; Lindsay et al., 1992; Runft et al., 2002; Went and Krause, 1974). A frequent response to the activating trigger in vertebrates and marine invertebrates is a rise in free calcium within the egg (reviewed in Lee et al., 2006; Parrington et al., 2007; Sato et al., 2006; Townley et al., 2006). In these organisms calcium acts as a second messenger to drive the downstream processes of egg activation. In insects, the requirement for calcium during egg activation has never been directly tested; however, recent reports show that a calcium-responsive regulator is essential for egg activation in *Drosophila melanogaster* (Horner et al., 2006; Takeo et al., 2006).

Drosophila egg activation, as in other insects that have been examined, is independent of fertilization. Unfertilized laid eggs can complete meiosis (Doane, 1960), modify their vitelline membranes (Heifetz et al., 2001; LeMosy and Hashimoto, 2000), and translate some maternal RNAs (Macdonald and Struhl, 1986) while degrading others (Tadros et al., 2003). Thus, *Drosophila* sperm trigger none of the traditional metrics of egg activation. Instead, activation initiates during ovulation (Heifetz et al., 2001) but the activating signal itself remains unknown.

One hypothesis for the activating signal in *Drosophila* derives from studies of Hymenoptera in which embryo development is triggered by oviposition. It has been proposed that mechanical stress imparted upon the egg during passage through the ovipositor is the signal that starts development in Hymenoptera. For instance, in the haplodiploid wasp, *Pimpla turionellae*, the diameter of the ovipositor is about one-third of the width of the egg, suggesting that physical deformation during egg laying initiates development. Consistent with this

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hypothesis, when *P. turionellae* eggs are dissected from the ovary and squeezed through a narrow capillary tube, over 70% of eggs activate, as measured by their ability to develop into haploid male larvae (Went and Krause, 1974). Pressure exerted on the egg can also activate eggs of another wasp, *Nasonia vitripennis*, as 23% of eggs dissected from the ovary and pressed with a needle were able to develop to larvae (King and Rafai, 1970).

The hypothesis that mechanical stimulation could also trigger *Drosophila* egg activation was initially suggested by two observations. First, Mahowald et al. (1983) briefly commented that application of hydrostatic pressure of an unspecified level or duration to *Drosophila* oocytes resulted in an increase in nuclear number in those oocytes. Whether such oocytes had properly completed meiosis and were undergoing haploid mitotic divisions was not reported. Second, Endow and Komma (1997) reported that pulling manually on the dorsal chorionic appendages of *Drosophila* oocytes triggered the resumption of meiosis in 3/3 cases. These intriguing observations suggested that mechanical stimulation might trigger the resumption and completion of meiosis, one aspect of egg activation. No other aspects of egg activation in response to mechanical stimulation were examined in those studies.

Analogous to oviposition in wasps, a mechanical trigger might occur as *Drosophila* eggs move from the ovary into the narrow lateral oviduct during ovulation. Mechanical stimulation could rearrange egg contents, leading to new structural or molecular combinations. Alternatively, mechanical stimulation could stimulate a mechanically-gated (MG) process, such as the opening or closing of stretch-activated (SA) ion channels. Such alterations to ion channels could lead to ionic changes analogous to those that trigger egg activation in other metazoans.

Another potential activation trigger in *Drosophila* is hydration. Mature oocytes in the ovary appear desiccated, whereas laid eggs are taut and expanded. Some evidence suggests that the hydrated contents of the oviduct lumen are transferred to eggs during ovulation (Mahowald et al., 1983). Support for a hypothesis that hydration could lead to egg activation is that incubation in a hypotonic buffer *in vitro* causes oocytes to swell and activate (Mahowald et al., 1983; Page and Orr-Weaver, 1997). Such hypo-osmotic swelling *in vivo* or *in vitro* could serve as another form of mechanical stimulation, by altering membrane tension to trigger a MG process (Martinac, 2004; Sachs, 1988). Additionally, specific ion(s) in the hydrating medium could provide the activation signal.

To better understand the activating signal in *Drosophila*, we wished to determine if pressure exerted on the egg effects activation. We found that external hydrostatic pressure accelerates activation, as assessed by vitelline membrane permeability changes and protein translation. In addition, an inhibitor of MG processes was able to inhibit hypo-osmotically induced activation, suggesting for the first time that the mechanism by which hydration leads to activation is through a MG response triggered by osmotic pressure. We also found that external calcium is necessary for both hypo-osmotic and pressure-accelerated activation. Therefore we demonstrate that the phenomenon of calcium-dependent egg activation extends to a new and important class of metazoans: insects. Taken together, these results suggest that mechanical stimulation from hydration and/or physical deformation during ovulation triggers activation in *Drosophila* by causing an influx of calcium into the egg. *Drosophila* is now poised to join organisms traditionally used to study activation, with the advantage of valuable genetic resources to discover the likely conserved pathways that mediate egg activation.

Materials and methods

Drosophila strains

D. melanogaster stocks were raised on yeast–glucose–agar medium at 23±2 °C in a 12-h L:12-h D photoperiod. The P2 strain of Oregon R was our wild-type (Allis et al., 1977).

In vitro egg activation

Eggs were activated *in vitro* by a procedure modified from Page and Orr-Weaver (1997). Virgin females were aged on yeasted food for 3–5 days. Mature stage 14 oocytes were dissected from ovaries in hypertonic Isolation Buffer (IB), which does not activate eggs. Oocytes were then incubated in 1–5 changes of hypotonic Activation Buffer (AB) for 5 min each. Activated oocytes were selected in 50% bleach (see below), or allowed to age for an additional period in Zalokar's Buffer (ZB), a physiological buffer that can support development (Page and Orr-Weaver, 1997). In experiments involving inhibitors we substituted an equimolar amount of PIPES for NaH_2PO_4 and KH_2PO_4 in AB and ZB because certain physiological anions such as phosphate can bind free gadolinium (see below), effectively removing it from solution (Caldwell et al., 1998). In addition, we found that calcium phosphate did not precipitate from AB made with PIPES, adding to the stability of AB. The PIPES-modified buffers performed identically to the original buffers from Page and Orr-Weaver (1997) (data not shown).

Vitelline membrane hardening

Assays were performed exactly as described in Horner et al. (2006). Briefly, activated eggs reorganize and cross-link proteins within their vitelline membranes, becoming impermeable to small molecules such as bleach (Heifetz et al., 2001; LeMosy and Hashimoto, 2000). Unactivated oocytes are permeable to bleach and lyse within 2 min of exposure to 50% bleach. Eggs activated *in vitro* were placed in 50% bleach for 2 min, and the number of resistant and lysed eggs was recorded.

Hydrostatic pressure

50–100 mature oocytes were dissected from females in IB. IB was replaced with AB, and approximately 10 mL of oocytes in AB were transferred to a standard 35 mL 20,000 psi FRENCH® Pressure Cell Body (Thermo-Spectronic, Rochester, NY), which was sealed shut by inserting the closure plug to force air from the cylinder, and closing the flow valve. The Pressure Cell was placed on the FRENCH® Press (Thermo-Spectronic), which is a hydraulic press that uses a motor-driven pump to vary hydrostatic pressure within the Pressure Cell. The pressure level was adjusted using a set of control valves, and pressure was applied for 7 min. Oocytes were removed from the Pressure Cell by pipetting through the re-opened closure plug.

RT-PCR

To obtain 0–2 h embryos, virgin females were aged on yeasted vials for 3–5 days and then mated to males the night before embryo collection. Mated females were allowed to deposit eggs on Petri plates containing grape juice agar for 2-h periods. The first collection was discarded, to eliminate

any eggs that were retained in the uterus overnight and thus had developed beyond the 2-h period. Eggs were washed off the plates in egg wash buffer (Karr and Alberts, 1986) and dechorionated in 50% commercial bleach (2.5% sodium hypochlorite). Total RNA was extracted from 300–350 embryos using Trizol/chloroform (Invitrogen, Carlsbad, CA). Non-activated mature oocytes were obtained by dissecting whole ovaries from 25 females in IB, and RNA was extracted as above. Approximately 10 μg RNA from each sample underwent cDNA synthesis using SuperScript™ II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. A 30 cycle PCR amplification was performed with the following conditions: 94 °C 2 min, 94 °C 45 s, 55 °C 45 s, 72 °C 1.5 min, 72 °C 10 min. PCR products were run on 1% agarose gels and DNA stained with 1 μg/mL ethidium bromide (Sigma-Aldrich, St. Louis, MO).

P-element excision

EY12268 (Bloomington stock # 21376) is an insertion of the Epgy2 *P* element on the third chromosome, 31 nucleotides upstream of the 5′ UTR of *ripped pocket (rpk)* (Bellen et al., 2004). This insertion was mobilized by crossing EY12268 flies to flies containing the Δ2,3 transposase on the third chromosome (*In(1)w^{m4h}*; *Df(3L)Ly*, *sens$^{Ly\text{-}1}$*/TM3, *ry^{RK} Sb^{1} Ser^{1}* P{Δ2-3}99B; Bloomington stock #2030). Jumpstarter males (EY12268/TM3, *ry^{RK} Sb^{1} Ser^{1}* P{Δ2-3}99B) obtained from this cross were crossed to *w^{1118}*; +/TM6, *Dr* females, and progeny were screened for P-element excision by loss of the mini-white marker. White-eyed male progeny were individually crossed to females carrying a deficiency that uncovers the *rpk* region (*w^{1118}*; *Df(3R)ED5092*, P{3′.RS5+3.3′} ED5092/TM6C; Bloomington stock #8091). Precise and imprecise excisions were identified by PCR of genomic DNA using primers that flanked the original insertion. If no product was detected, primers were used to amplify the *rpk* coding region from genomic DNA to detect possible genomic deletions, and cDNA, to detect disruption of *rpk* expression.

Inhibitor treatment

Gadolinium

$GdCl_3 \cdot 6H_2O$ (Sigma-Aldrich) was added to buffers at a final concentration of 50, 100 or 200 μM immediately before use, and oocytes were activated as described above. Control buffers without $GdCl_3$ were made in parallel.

BAPTA and EGTA

Ca^{2+} was buffered below 50 nM by the addition of BAPTA [1,2-Bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrapotassium salt] (Sigma-Aldrich) at final concentrations of 5, 10 and 11 mM in IB, AB and ZB, respectively (as calculated by MaxChelator; Bers et al., 1994). Control buffers without BAPTA were made in parallel. Sucrose concentration in control buffers was adjusted so the final osmolarity matched the solutions containing BAPTA. To detect if this osmolarity increase alone affects hypo-osmotic activation, an additional control AB without added sucrose was also used.

Since EGTA [Ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] (Sigma-Aldrich) does not buffer Ca^{2+} as well as BAPTA, we could not lower extracellular Ca^{2+} to the same level (50 nM) without greatly affecting osmolarity and thus, potentially, activation. Therefore, Ca^{2+} was buffered below 350 nM by the addition of EGTA at a final concentration of 5 and 25 mM in IB and AB/ZB, respectively (as calculated by MaxChelator; Bers et al., 1994). The controls for EGTA were analogous to those for BAPTA.

Immunoblotting

Samples were lysed in ~20 μl protease-inhibiting homogenization buffer, to which an equal amount of SDS sample buffer was added (Monsma and Wolfner, 1988). Samples containing 30–40 μg of protein were electrophoresed on 7.5% polyacrylamide SDS gels and subjected to Western blotting analysis as in Lung and Wolfner (1999) with anti-SMAUG (SMG) antibody (kindly provided by W. Tadros, University of Toronto; Smibert et al., 1999) at a 1:10,000 dilution. As a loading control, anti-α-tubulin (Sigma-Aldrich) was used at a 1:20,000 dilution. Secondary antibody was visualized with the ECL chemiluminescence system (Amersham Biosciences, Piscataway, NJ). Signal intensity was quantified using Quantity One (Bio-Rad, Hercules, CA), adjusted for background and normalized to tubulin.

Immunofluorescence

Eggs activated *in vitro* were fixed and stained exactly as described in Horner et al. (2006). Briefly, activated eggs were dechorionated in 50% bleach, fixed in Methanol/Heptane, and stored at 4 °C until use. Fixed eggs were rehydrated in phosphate-buffered serum (PBS) containing 0.1% TritonX-100 (Karr and Alberts, 1986) by washing three 3 times for 5 min each, and then permeabilized by washing a further 45 min in PBST. Eggs were incubated overnight at 4 °C in PBST containing a monoclonal anti-α-tubulin (mouse) antibody (clone B-5-1-2, Sigma-Aldrich) at a dilution of 1:200 and RNaseA (Roche Applied Science, Indianapolis, IN) at a concentration of 5 μg/mL. Primary-antibody bound eggs were incubated with Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies (Invitrogen) for 2 h at room temperature. DNA was stained with 10 μg/μL propidium iodide (Invitrogen) for 10 min, and eggs were mounted on glass microscope slides in 75% glycerol containing 940 mM *n*-propyl gallate.

Statistics

Logistic regression was used to test for an effect of hydrostatic pressure on VM hardening. Chi-square analysis was used to compare the effects of treatments, with $P<0.05$ considered significant.

Results and discussion

In vitro activation leads to a progressive increase in vitelline membrane hardening, protein translation and meiosis completion

Drosophila oocytes are activated *in vitro* using two buffers in sequence: (1) "Isolation Buffer" (IB), a hypertonic solution in which oocytes can be prepared, but in which they do not activate, and (2) "Activation Buffer" (AB), a hypotonic solution in which oocytes undergo most activation events (Mahowald et al., 1983; Page and Orr-Weaver, 1997). To establish the conditions for our subsequent tests of effects of hydrostatic pressure on egg activation, we determined the time course of *in vitro* activation at normal atmospheric pressure. We dissected mature oocytes from wild-type females in IB, and then incubated them in AB for 5, 10, 15, 20, or 25 min. The degree of activation was first assayed by measuring oocytes' resistance to lysis by bleach [a consequence of reduced permeability to small molecules as proteins in the vitelline membrane (VM) become cross-linked during activation (Heifetz et al., 2001; LeMosy and Hashimoto, 2000)]. We found that VM hardening increased gradually and progressively over time (Fig. 1A).

Another feature of activation is the translation of certain maternal transcripts; 8 are currently known, including *smaug* (*smg*) (Macdonald and Struhl, 1986; Tadros and Lipshitz, 2005). Translation of *smg* is repressed in mature oocytes by PUMILIO and at least one other repressor, but upon egg laying and activation this repression is relieved by PAN GU kinase (Tadros et al., 2007). To determine if *smg* is translated upon *in vitro* activation, mature oocytes were dissected in IB, incubated in AB for 15 min (to be comparable to experiments below), and aged in a physiological buffer (Zalokar's buffer, ZB) for 0, 30, or 60 min. SMG protein was first detected in eggs aged for

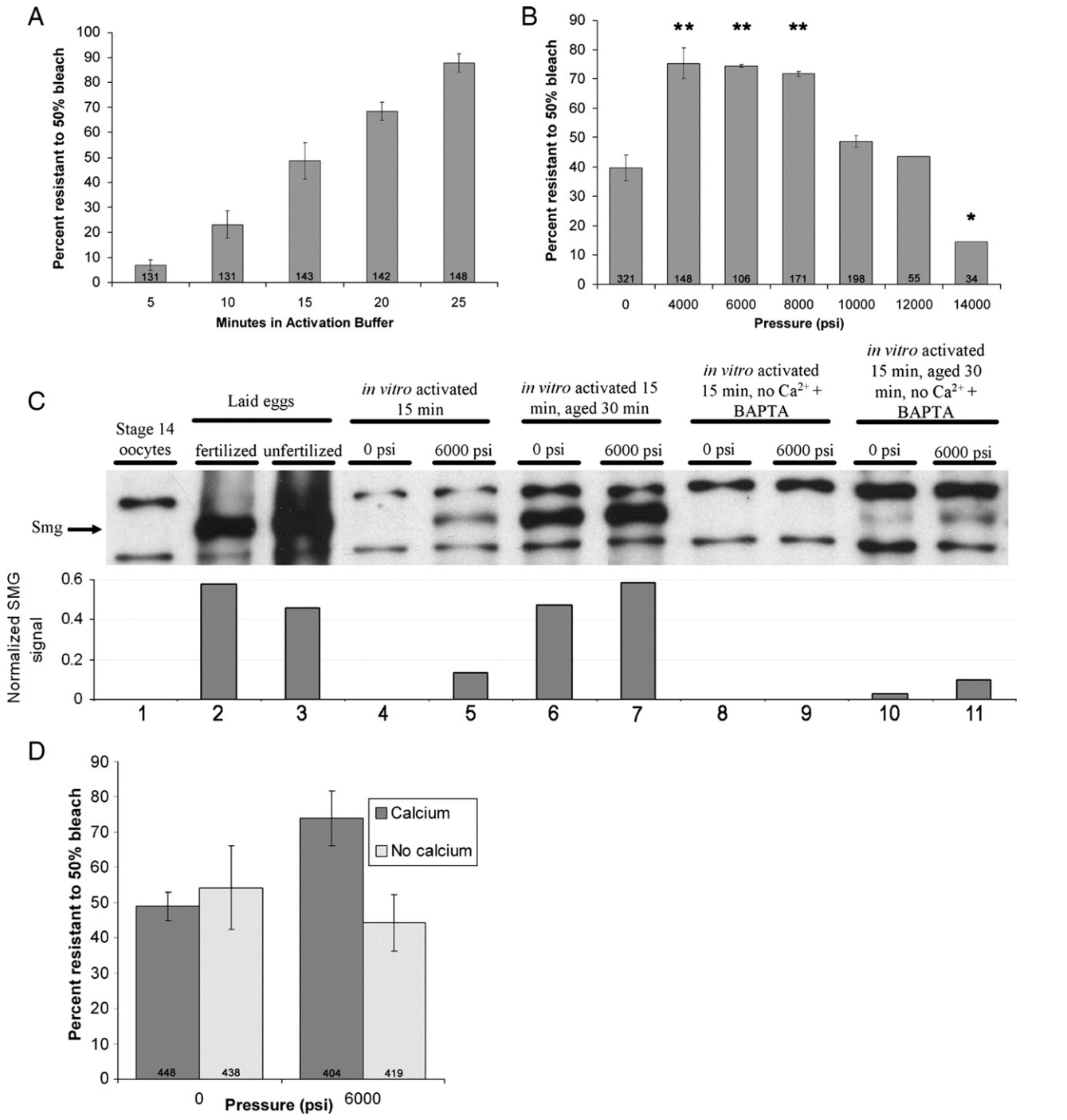

Fig. 1. Hydrostatic pressure accelerates VM hardening and protein translation in a Ca^{2+}-dependent manner. (A) VM hardening increases gradually during *in vitro* activation. (B) Hydrostatic pressure significantly affects VM hardening (Logistic regression, $\chi^2_7 = 72.5$, $P < 0.0001$). $^{**}P \leq 0.001$, $^{*}P \leq 0.01$ in post-hoc Chi-square analysis between 0 psi and 4000, 6000, 8000, and 14,000 psi ($\chi^2_1 = 53.0$, 37.6, 45.0, and 8.5, respectively). (C) Hydrostatic pressure accelerates SMG translation in a Ca^{2+}-dependent manner. Graph corresponds to lanes shown directly above; values from scans were background adjusted, normalized to tubulin and averaged over two independent experiments. (D) Hydrostatic pressure increases VM hardening only when external Ca^{2+} is added to the buffers (compare dark bars to light bars). In panels A, B and D the numbers in bars indicate the number of eggs used in each experiment.

30 min, and this level increased at 60 min (Fig. 1C, lanes 4 and 6, and Supplementary Fig. 1).

Mature *Drosophila* oocytes in the ovary are arrested at metaphase of meiosis I, with a mass of unindividualized chromatin in the middle of a single bipolar spindle (King, 1970; Page and Orr-Weaver, 1997). During *in vivo* egg activation, ovulation triggers the resumption of meiosis, and the two meiotic divisions occur as the eggs pass through the female reproductive tract (Heifetz et al., 2001). A previous study showed that meiosis also resumes and completes during *in vitro* egg activation (Page and Orr-Weaver, 1997). In that study the eggs were placed in AB for a short pulse, and then incubated in

ZB for at least 25 min. Under these conditions, the majority of eggs (53%) had 3–4 meiotic products and thus had completed meiosis II. Since most of our subsequent experiments would involve shorter, 15-min, activations, we wished to determine meiotic progression up to this time point. We therefore activated eggs *in vitro* for 15 min, and fixed and stained them with antibodies against tubulin to visualize the spindle, and propidium iodide to observe the DNA. After 15 min in AB, the majority of eggs (67.7%) were undergoing either meiosis I or meiosis II (Supplementary Fig. 2 and Supplementary Table 1). Eggs that had resumed meiosis I (32.4%) contained a single spindle in which the chromatin had individualized into distinct chromosomes and/or was being pulled toward opposite poles of the spindle. Eggs undergoing meiosis II (35.3%) were identified by the presence of two tandemly oriented spindles (Supplementary Fig. 2A). A smaller proportion (29.4%) of eggs contained 4 meiotic products and thus had completed meiosis II (Supplementary Figs. 2B, C). In a single egg the meiotic products had fused into a rosette-structured polar body, marking the completion of meiosis (Supplementary Fig. 2D).

External hydrostatic pressure accelerates VM hardening and protein translation in vitro

To test if hydrostatic pressure has an effect on egg activation, we applied hydrostatic pressure to mature oocytes in AB (see Materials and methods) and assayed VM hardening using bleach resistance. To detect increases or decreases in activation, the total time in AB was limited to 15 min, the time at which approximately 50% of the eggs under atmospheric pressure attain bleach resistance (Fig. 1A). Hydrostatic pressure was applied for 7 of the 15 min, based on a time-course we performed (data not shown) as well as previous reports (Held, 2003; Srdic and Jacobs-Lorena, 1978). Mature oocytes dissected from females were subjected to pressures in the range of 4000–14,000 pounds per square inch (psi). There was an overall significant effect of hydrostatic pressure on VM hardening (Logistic regression, $P<0.0001$) (Fig. 1B). Oocytes responded to increasing hydrostatic pressure with an increase in bleach resistance that peaked at 5500 psi (Logistic regression analysis) and decreased sharply at pressures above 8000 psi. Post-hoc chi-square analyses revealed that there was a significant increase in VM hardening when eggs were exposed to 4000, 6000, and 8000 psi ($P \leq 0.001$).

To test for effects of pressure on protein translation, we applied 6000 psi to oocytes as above, which is as close to the peak of the VM hardening curve as can be accurately measured. Control oocytes were activated in parallel at atmospheric pressure. In a 15-min activation, oocytes exposed to 6000 psi translated SMG, whereas those without external pressure did not (Fig. 1C, lanes 4 and 5). Thus, hydrostatic pressure accelerates SMG translation. When oocytes were allowed to age in ZB for 30 min after the activation, SMG protein is detected at comparable levels in both pressurized and unpressurized oocytes, indicating that with time SMG translation can reach equivalent levels (Fig. 1C, lanes 6 and 7).

Another assay for activation is the completion of meiosis. However, a similar level (5000 psi) of hydrostatic pressure has been used as an effective polyploidizing agent in both *Rana pipiens* oocytes and *Drosophila* embryos, due to disorganization of the mitotic spindle that inhibits chromosome segregation during anaphase (Dasgupta, 1962; Held, 2003). We found, similarly, that our conditions disrupted the spindles, causing many to appear unfocused at one or both poles, or to become tripolar (Supplementary Fig. 2F). In addition, chromosomes occasionally escaped from the main spindle and nucleated their own tiny spindle (Supplementary Fig. 2G). These abnormalities presumably prevented the normal completion of meiosis, as only two eggs appeared to be undergoing an abnormal meiosis II and no eggs were observed with four meiotic products (Supplementary Table 1). In the two rare cases where a polar body was observed, indicating the completion of meiosis, smaller secondary spindles were also present (Supplementary Fig. 2H). Thus, under our conditions we were unable to use meiosis to assay egg activation.

Gadolinium inhibits hypo-osmotically induced activation

The results above indicate that oocytes are sensitive to external pressure applied uniformly and directly to the membrane. Another type of mechanical stimulation is hypo-osmotic swelling (Hamill and McBride, 1996), which occurs during both *in vivo* and *in vitro* egg activation in *Drosophila*. Hypo-osmotic swelling could theoretically alter membrane tension to trigger a MG process, such as activation of SA ion channels. To test this, we used a potent inhibitor of MG processes, gadolinium ($GdCl_3$), which blocks SA ion channels in *Xenopus* oocytes (Yang and Sachs, 1989). To act as an effective inhibitor of SA ion channels, the concentration of $GdCl_3$ should not be too high or too low; at low concentrations it may stimulate ion channel activity (Hamill and McBride, 1996), and at high concentrations it may be non-specific (Lansman, 1990). Acceptable ranges are 10–1000 μM (Hamill and McBride, 1996), with most vertebrate SA ion channels blocked at 30–50 μM. Therefore, we initially performed our experiments using two concentrations of $GdCl_3$ at the low- to mid-level, 50 μm and 100 μM. Mature oocytes dissected from wild-type *Drosophila* females were incubated in buffers with or without $GdCl_3$, and activation was assessed by bleach resistance and translation of SMG. In three independent experiments, we found that both 50 μM and 100 μM $GdCl_3$ significantly reduced the percentage of oocytes that underwent VM hardening (Table 1 and Fig. 2A). In addition, 200 μM $GdCl_3$ inhibited the translation of SMG (Fig. 2D, lanes 6 and 7). Together these results suggest that hypo-osmotic swelling triggers activation via a MG response.

One can estimate the amount of osmotic pressure experienced by oocytes during *in vitro* hypo-osmotic activation. There is an approximately 200 mosM difference between IB and AB, which converts to an osmotic pressure across a semi-permeable barrier of 69.6 psi, or 4.8×10^6 dyn/cm^2 (Kung, 2005). If the surface area of a *Drosophila* egg is 6.3×10^{-4} cm^2, this osmotic

Table 1
Gadolinium inhibits hypo-osmotically inducted activation

Experiment (15 minutes in AB)	No gadolinium	100 μM gadolinium	No gadolinium	50 μM gadolinium
1. Resistant/Total # oocytes	36/83 (43.4)	4/97 (4.1)**	15/34 (44.1)	3/39 (7.7)**
2. Resistant/Total # oocytes	21/51 (41.2)	2/86 (2.3)**	13/29 (44.8)	3/35 (8.6)**
3. Resistant/Total # oocytes	80/137 (58.4)	4/77 (5.2)**	32/59 (54.2)	1/60 (1.7)**
Sum Resistant/Total	137/281 (48.8 ± 5.4)	10/260 (3.9 ± 0.85)	60/122 (49.2 ± 3.3)	7/134 (5.2 ± 2.2)

Oocytes were activated *in vitro* for 15 min with or without $GdCl_3$, and VM hardening was assayed by bleach resistance. Numbers in parentheses indicate percentages, and where appropriate ± S.E.M. $^{**}P \leq 0.001$ as per Chi-square test.
1. 100 μM: $\chi^2_1 = 39.9$, $P \leq 0.001$, 50 μM: $\chi^2_1 = 12.9$, $P \leq 0.001$.
2. 100 μM: $\chi^2_1 = 34.6$, $P \leq 0.001$, 50 μM: $\chi^2_1 = 11.1$, $P \leq 0.001$.
3. 100 μM: $\chi^2_1 = 58.5$, $P \leq 0.001$, 50 μM: $\chi^2_1 = 41.0$, $P \leq 0.001$.

pressure would generate approximately 3024 dyn/cm stretch force on the surface of the egg. The amount of pressure required for half activation of most known SA channels is ~several dyn/cm (Martinac, 2004). Even if the transmembrane difference of *in vitro* activated eggs is less than 200 mosM/L it is still likely that there is sufficient osmotic pressure to stimulate the opening of SA ion channels during hypo-osmotic activation.

Expression of putative SA ion channels

Two broad classes of ion channel families have been shown in a variety of organisms to respond to external stimuli: the transient receptor potential (TRP) superfamily and the degenerin/epithelial Na^+ channel (DEG/ENaC) superfamily. The *Drosophila* genome contains 13 members of the TRP channel superfamily (reviewed in Montell, 2005). Of these, four appear to be activated through mechanical stimulation. Two, *nanchung*, and *inactive*, are TRPV cation channels that open in response to osmolarity changes (Gong et al., 2004; Kim et al., 2003). Two other TRP family members, *NompC* (TRPN) and *painless* (TRPA), respond to light touch and noxious thermal and mechanical stimuli, respectively (Tracey et al., 2003; Walker et al., 2000).

We performed RT-PCR to determine if RNAs encoding these proteins are present in *Drosophila* ovaries or 0–2 h embryos. We did not detect *nanchung*, *inactive*, or *NompC* RNA in ovaries or 0–2 h embryos (Figs. 3A, B). However, *painless* RNA was detected in ovaries (but not 0–2 h embryos; Fig. 3B). A null *painless* mutation is unfortunately not available to test its potential role in egg activation.

The other broad class of ion channels involved in sensory perception is the DEG/ENaC family, originally identified in *C. elegans* genetic screens for mutants defective in mechanosensation

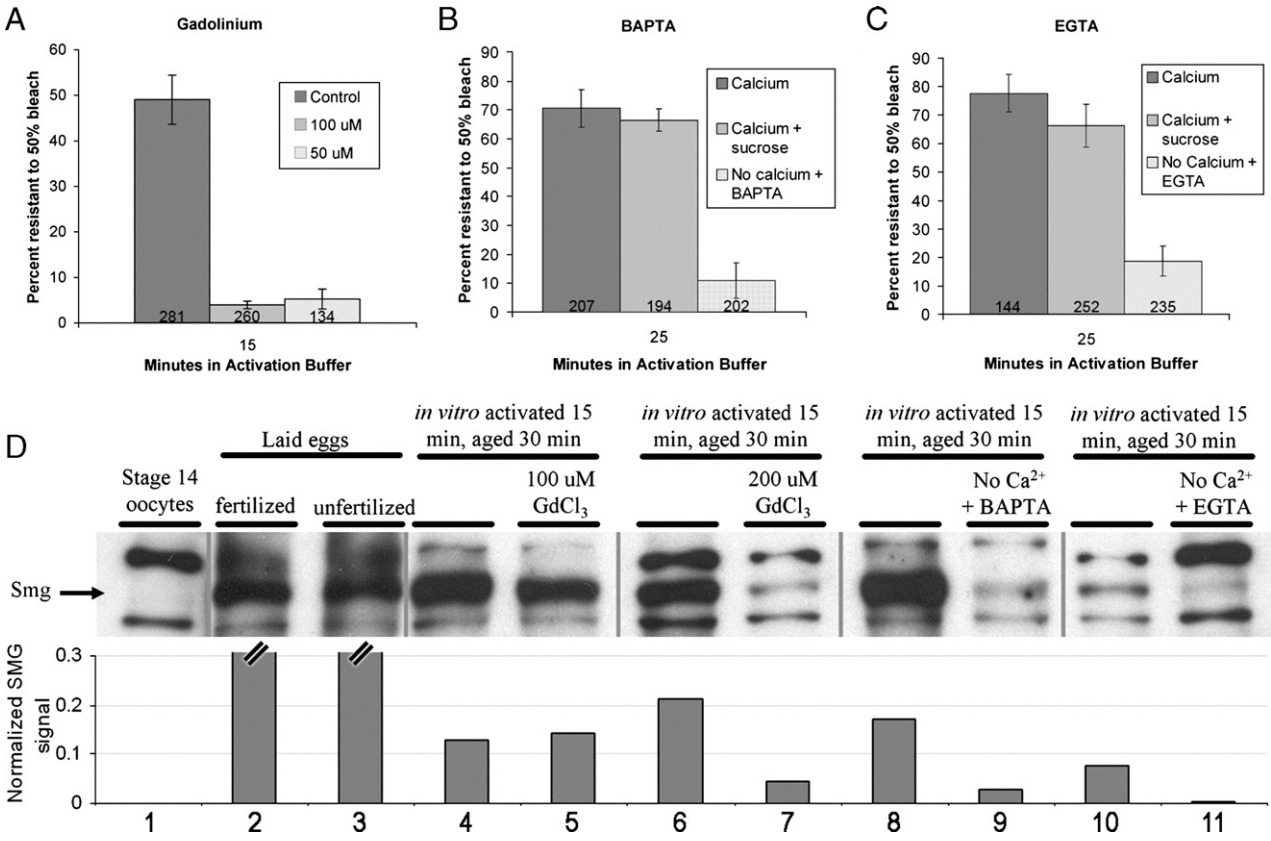

Fig. 2. Hypo-osmotic swelling generates a mechanical response that requires external Ca^{2+}. (A) $GdCl_3$, (B) BAPTA, and (C) EGTA significantly decrease VM hardening. The numbers in bars indicate the number of eggs used in each experiment. (D) $GdCl_3$, BAPTA, and EGTA reduce SMG translation. Lines between lanes indicate separate experiments. Graph corresponds to lanes shown directly above; values from scans were background adjusted, normalized to tubulin and averaged over two experiments. Hatch bars indicate values greater than graph range (0.77 fertilized and 0.78 unfertilized).

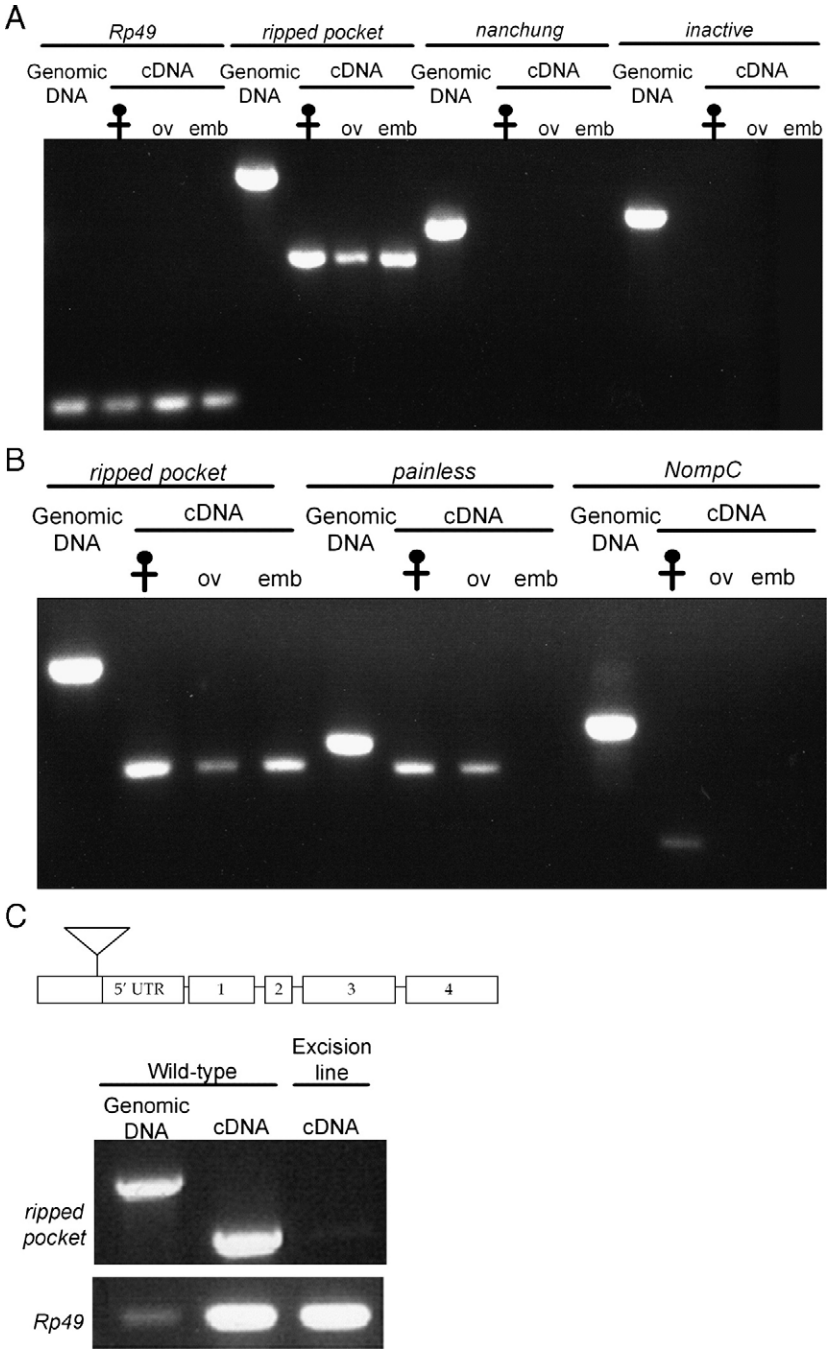

Fig. 3. Expression of putative SA ion channels in *Drosphila* oocytes and early embryos. (A) *ripped pocket* mRNA is expressed in whole females, ovaries, and 0–2 h embryos. *nanchung* and *inactive* mRNAs are not expressed in ovaries or 0–2 h embryos; however, expression was not detected in whole females, so it is possible that this method is not sensitive to very low-level expression. *Rp49* (Ribosomal protein 49) served as an internal control for genomic DNA and cDNA preparation. (B) *painless* mRNA is expressed in whole females and ovaries, but not 0–2 h embryos. *NompC* mRNA is not expressed in ovaries or 0–2 h embryos. The internal control in this experiment was *ripped pocket*. (C) (Top) Line EY12268 contains a *P* element insertion (triangle) 31 nucleotides upstream of the 5′UTR of *rpk*. Exons 1–4 of *rpk* are numbered. (Bottom) In one imprecise excision line, the expression of *rpk* in ovaries is lowered compared to WT ovaries.

(Chalfie and Au, 1989; Chalfie and Sulston, 1981). There are 25 candidates in the *Drosophila* genome, 16 of which have been characterized (Adams et al., 1998; Liu et al., 2003). The majority (15 of the 16) are *pickpocket (ppk)* genes that are not expressed in ovaries or early embryos (Liu et al., 2003). One, *ripped pocket (rpk)* is expressed in ovaries and 0–2 h embryos (Fig. 3A and Adams et al., 1998). RPK is a putative SA Na^+ channel, based on its sensitivity to $GdCl_3$ and its ability to form a transduction channel when expressed in *Xenopus* oocytes (Adams et al., 1998).

A null *rpk* mutant is not available. However, a fly strain carrying a P element insertion 31 nucleotides upstream of the *rpk* 5′ untranslated region (5′UTR) exists, and we obtained this strain (Fig. 3C; Bellen et al., 2004). Unfortunately, we

found that this *P* element does not disrupt expression of *rpk* (data not shown). We therefore mobilized the *P* element in an attempt to obtain excisions that could abolish or severely diminish *rpk* expression. We obtained 30 precise and 11 imprecise excisions. The coding region of *rpk* was not affected in any of the imprecise excision lines (data not shown). However, since removal of upstream regulatory sequences could potentially affect expression of *rpk*, we performed RT-PCR experiments to examine whether any of the imprecise excisions decreased or abolished *rpk* RNA levels in ovarian oocytes. One of the 11 excisions showed a drastic decrease in *rpk* RNA levels in oocytes, although very low levels were still detected (Fig. 3C). Thus this allele, although the strongest disruption we could obtain, is not a null. Homozygous females from this line were viable and fertile. The fertility of the homozygous mutant females suggests that severe disruption in RPK levels does not prevent egg activation. However, we cannot determine from these data whether the low levels of RPK remaining in the mutant were sufficient to permit egg activation, or if other channels are redundant with RPK in egg activation function. Future studies to generate null mutations in other candidate genes, and to test them for activation effects singly and in combination will be needed to address these issues.

External Ca^{2+} is required for hypo-osmotic activation

A well-documented response to osmotic swelling in animal cells is an increase in intracellular Ca^{2+} (Hamill and Martinac, 2001; Kim et al., 2003; Shen et al., 2003). This could result from Ca^{2+} influx from the external environment or Ca^{2+} mobilization from internal stores. Due to the established role of Ca^{2+} in egg activation of many organisms (see below), as well as recent evidence for involvement of a Ca^{2+}-responsive pathway in *Drosophila* activation (Horner et al., 2006; Takeo et al., 2006), we investigated whether external Ca^{2+} is required for hypo-osmotic activation. IB, AB, and ZB contain 1, 2, and 7 mM Ca^{2+}, respectively. Consistent with an earlier report (Mahowald et al., 1983) we found that simply omitting Ca^{2+} from the buffers did not impede hypo-osmotic activation (Fig. 1D, left pair of bars). However, this omission does not remove residual Ca^{2+} present at low levels.

We thus omitted Ca^{2+} and buffered any residual Ca^{2+} below 50 nM using the Ca^{2+} chelator BAPTA. In three independent experiments, we found that BAPTA significantly lowered the percentage of oocytes undergoing VM hardening when compared to the matched-osmolarity, sucrose-containing control (Table 2 and Fig. 2B). In each experiment there was no difference between controls with or without additional sucrose. In addition, we found that oocytes activated in the absence of external Ca^{2+} and with BAPTA were impaired in their ability to translate SMG (Fig. 2D, lanes 8 and 9).

To confirm these results and to eliminate possible non-specific effects of external BAPTA, we carried out similar experiments using a different Ca^{2+} chelator, EGTA. In four independent experiments, we found that oocytes activated in the absence of external Ca^{2+} and with EGTA underwent VM hardening at significantly lower levels than the matched-osmolarity controls containing Ca^{2+} (Supplementary Table 1 and Fig. 2C). These oocytes also translated SMG at lower levels than control (Fig. 2D, lanes 10 and 11).

Table 2
Oocytes activated in the absence of external Ca^{2+} and with BAPTA undergo VM hardening at significantly lower levels than controls

Experiment (25 minutes in AB)	Ca^{2+} + 0 mM	Ca^{2+} + sucrose	No Ca^{2+} + BAPTA
1. Resistant/Total # oocytes	44/72 (61.1)	45/65 (69.2)	14/59 (23.7)
		**	
2. Resistant/Total # oocytes	59/71 (83.1)	50/71 (70.4)	5/71 (7.04)
		**	
3. Resistant/Total # oocytes	43/64 (67.2)	34/58 (58.6)	3/72 (4.2)
		**	
Sum Resistant/Total	147/207 (70.5 ± 6.56)	129/194 (66.5 ± 3.75)	22/202 (10.9 ± 6.08)

Oocytes were activated *in vitro* for 25 min in the indicated buffers, and VM hardening assayed by bleach resistance. Numbers in parentheses indicate percentages, and where appropriate ± S.E.M. Brackets delineate comparisons, $^{**}P \leq 0.001$ as per Chi-square test.
1. $\chi^2_1 = 25.7$, $P \leq 0.001$.
2. $\chi^2_1 = 60.1$, $P \leq 0.001$.
3. $\chi^2_1 = 46.8$, $P \leq 0.001$.

Studies of egg activation in other organisms have established that increased free Ca^{2+} within the oocyte drives the downstream processes of activation (reviewed in Ducibella et al., 2006; Horner and Wolfner, 2008). The method by which Ca^{2+} increases within the oocyte upon activation varies between species. In vertebrates and some marine invertebrates, fertilization causes Ca^{2+} to be released from intracellular reserves in the endoplasmic reticulum. In marine bivalves such as *Mactra*, however, the Ca^{2+} wave that occurs upon fertilization starts from the entire oocyte cortex and spreads inward, suggesting an influx of external Ca^{2+} (Deguchi and Morisawa, 2003). Sperm do not exclusively trigger the activation-inducing Ca^{2+} wave, since exposure to seawater Mg^{2+} during spawning leads to an internal Ca^{2+} wave in the shrimp *Sicyonia ingentis* (Lindsay et al., 1992). Our results suggest that *Drosophila* oocytes respond to the sperm-independent activating trigger in part by allowing external Ca^{2+} to enter eggs. Therefore, while proximate triggers for egg activation might differ among metazoans, Ca^{2+}-dependence of egg activation mechanisms appears to be a general theme, as extended here for the first time to an insect.

External Ca^{2+} is required for pressure-accelerated activation

We also tested the requirement for external Ca^{2+} during hydrostatic pressure-accelerated activation. In the control condition, mature oocytes were dissected from wild-type females in IB containing Ca^{2+} and exposed to 6000 psi of hydrostatic pressure for 7 min in AB containing Ca^{2+}. In five independent experiments, we found that 6000 psi significantly accelerated VM hardening when Ca^{2+} was present in the buffers (Table 3 and Fig. 1D). Hydrostatic

Table 3
External Ca^{2+} is required for pressure-accelerated activation

Experiment	Ca^{2+} + 0 psi	Ca^{2+} + 6000 psi	No Ca^{2+} + 0 psi	No Ca^{2+} + 6000 psi
1. Resistant/Total # oocytes	32/88 (36.4) **a	51/78 (65.4)	39/92 (42.4)	19/65 (29.2)
2. Resistant/Total # oocytes	30/71 (42.3) **b	54/78 (69.2)	54/83 (65.1) *c	30/75 (40)
3. Resistant/Total # oocytes	46/86 (53.5) *d	61/86 (70.9)	49/87 (56.3)	46/93 (49.5)
4. Resistant/Total # oocytes	64/95 (67.4) *e	63/76 (82.9)	57/83 (68.7) *f	41/87 (47.1)
5. Resistant/Total # oocytes	49/108 (45.4) **g	71/86 (82.6)	40/93 (43)	43/99 (43.4)
Sum Resistant/Total	221/448 (49.3 ± 5.4)	300/404 (75 ± 3.6)	239/438 (54.6 ± 5.5)	179/419 (42.7 ± 3.5)

Columns 1 and 2: Oocytes in IB and AB containing Ca^{2+} and exposed to 6000 psi of hydrostatic pressure significantly increase VM hardening. Columns 3 and 4: Oocytes in IB and AB lacking Ca^{2+} and exposed to 6000 psi of pressure do not increase VM hardening. Numbers in parentheses indicate percentages, and where appropriate ± S.E.M. Brackets delineate comparisons, $^{**}P \leq 0.001$ or $^{*}P \leq 0.025$ as per Chi-square test.
[A] $\chi_1^2 = 13.9$, $P \leq 0.001$.
[B] $\chi_1^2 = 11.0$, $P \leq 0.001$.
[C] $\chi_1^2 = 9.9$, $P \leq 0.01$.
[D] $\chi_1^2 = 5.6$, $P \leq 0.025$.
[E] $\chi_1^2 = 5.3$, $P \leq 0.025$.
[F] $\chi_1^2 = 8.1$, $P \leq 0.01$.
[G] $\chi_1^2 = 28.1$, $P \leq 0.001$.

pressure also accelerated the translation of SMG (Fig. 1C, lanes 4 and 5). However, when Ca^{2+} was omitted from the buffers, exposure of oocytes to 6000 psi no longer accelerated VM hardening or the translation of SMG (in the former case, this was true even without BAPTA or EGTA) (Fig. 1C, lanes 8 and 9, Fig. 1D).

Hydrostatic pressure might accelerate activation by stimulating SA ion channels. In mammals, an epithelial sodium channel opens in response to hydrostatic pressure in a Ca^{2+}-dependent manner (Ghazi et al., 1998). However, SA ion channels might only respond to membrane tension along the plane of the cell membrane, not to hydrostatic pressure perpendicular to it (Martinac, 2004). Another mechanism by which hydrostatic pressure might accelerate activation is non-specific permeabilization of the plasma membrane to Ca^{2+}. High hydrostatic pressure can cause transient permeabilization of *Escherichia coli* membranes (Ulmer et al., 2002). In *Drosophila* oocytes, non-specific permeabilization of the plasma membrane to Ca^{2+} could accelerate activation analogously to the artificial activation of marine invertebrates and vertebrates using ionophores to cause an influx of Ca^{2+} (Schuetz, 1975; Steinhardt and Epel, 1974; Uehara and Yanagimachi, 1977). These experiments suggest that hydrostatic pressure allows Ca^{2+} to enter eggs, and this influx of Ca^{2+} significantly accelerates VM hardening and SMG translation.

In summary, we have shown that despite the difference in upstream trigger for activation, *Drosophila* oocytes, like those of many other animals, require Ca^{2+} for downstream activation events. Our results also show that external Ca^{2+} appears to enter *Drosophila* oocytes through a mechanosensitive process stimulated by pressure on the oocytes and/or swelling of the oocytes as they pass through the *Drosophila* reproductive tract. At least two potential SA ion channels are expressed in *Drosophila* oocytes; a complete characterization of all putative SA ion channels will likely discover more. Future studies can take advantage of *Drosophila*'s genetic resources to test the role of these SA ion channels in insect egg activation. However, our finding that calcium is required for downstream activation events, as it is in all other organisms studied, means that *Drosophila* genetics can also be applied to the discovery and characterization of conserved calcium pathways during egg activation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.01.014.

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